IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of
MIAN et al.
Serial No. 10/533,935
Title: Quantifying Exposure to Stress

DECLARATION

The Hon. Commissioner For Patents
PO Box 1450
Alexandria.VA 22313-1450

Dear Sir:

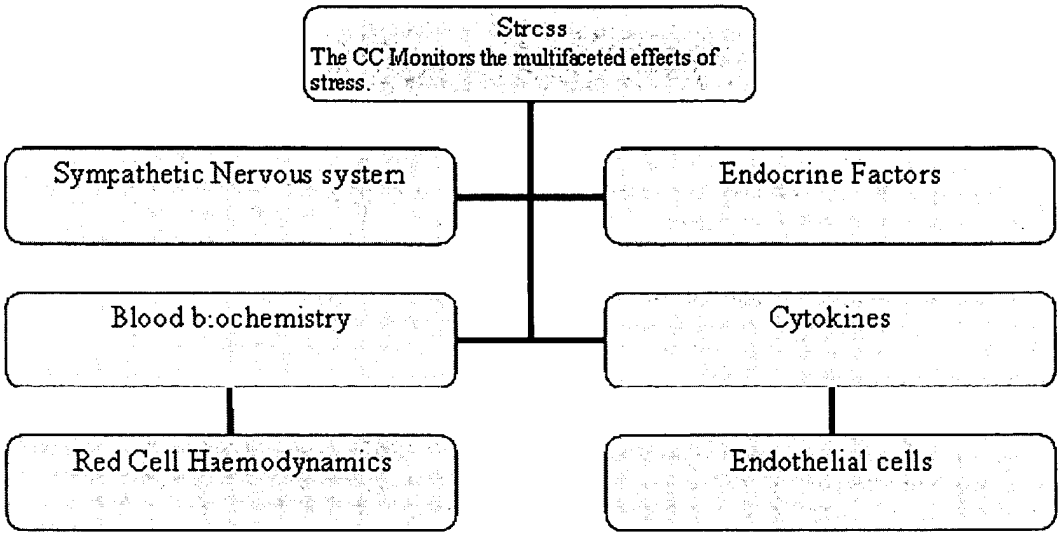
I, Dr Rubina Mian, do hereby declare and state as follows:

1. I am a named inventor for the above-identified application. I have read the Final Office Action dated 03/12/2007 and the subsequently issued Office Action (now withdrawn). I have also read the documents cited by the Examiner. I wish to correct a number of misunderstandings I see in the arguments of the Examiner relying on the Tsukamoto et al. and Mikawa et al. papers. In doing so, I will also say more about the technical features of the claimed method and its importance in my field of academic research and more generally.

2. I hold a PhD in Physiology from the University of Birmingham one of the top UK universities.This high international reputation is reflected by a 5 (one of the highest categories) awarded in the 2001 Research Assessment Exercise (carried out by the Higher Education Funding Council for England). I also hold a B.Sc. (Hons.) Pharmacology from the University of Liverpool (equally internationally rated). Relevant experience includes: 22 years research on mechanisms of leukocyte activation in an industrial and academic environment. I worked as a research scientist for Miles Laboratories, which was then taken over by Bayer AG. I was a research fellow at the University of Birmingham, and have worked as an honorary research associate at the Wildlife Conservation Research Unit, Oxford University, UK, and am currently a senior lecturer at Coventry University. I have won numerous awards including the prestigious Medici Fellowship. I have supervised PhDs in stress research. Relevant PhD projects have included research into psychological stress and physiological mechanisms; developing novel techniques for measuring stress and researching mechanisms of cellular activation in stress. I was one of three UK

scientists to be invited to develop strategy for the European Space Agency's 'Future Programme in Life Sciences'. Amongst various international invitations, my team was the only UK team to be invited to contribute to a book chapter in 'Stress and Health' - a leading work in the field. I have been an invited peer reviewer of grant submissions to: the European Union, Advantage West Midlands and The Medical Research Council, UK and reviewed articles for the Scandinavian Journal Of Immunology, Biological Conservation Journal of Applied Physiology, Journal of Zoology, Circulation Research, Medical Science Monitor (online) and the American Journal of Physiology. In 2000, I was part of a team that won The (UK) National Health Service (NHS) Equality Award for Outstanding Achievement. I have served as a non-executive director of an NHS trust (appointed directly by the UK Secretary of State) for 5 years.

3. The test of the invention monitors the multifaceted effects of stress using the body's leukocytes (primarily, but not exclusively, neutrophils) as bio-indicators. These cells circulate throughout the body picking up and responding to all of the signals of stress (indicated by the diagram below). Leukocytes (primarily, but not exclusively, neutrophils) have over 150 different receptors which can respond to a diverse range of factors, all of which are sensitive to stress. These include: endocrine factors in the plasma, changes in blood biochemistry, changes in red cell haemodynamics, cytokines and factors released from other cells, both circulating and non-circulating cells such as endothelial cells, and changes in the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system. As stress affects each of these factors, leukocytes make ideal indicators of stress, being constantly exposed to a diverse range of stress stimuli. The Coping Capacity of leukocytes (CC), i.e. their ability to respond to an external stimulator and produce reactive oxygen species, will be affected by the immediate external environment in the blood. Leukocytes (mainly neutrophils) which have been exposed to stressors within the body will have a reduced capacity to produce reactive oxygen species in response to an external stimulator (e.g. PMA). This is the underlying technical foundation of the invention.



4. The test of the invention is as far as I am aware the first test to provide a physiologically relevant blood test for objectively assessing the effect of stress. The physiological relevance is convincing since:

i) We deliberately keep the leukocytes in the local environment, i.e. we keep them suspended in the blood. The suspension of leukocytes in blood allows the cells to dynamically interact with the surrounding red cells and allows cell-cell interaction within and between different leukocyte cohorts. This can dramatically affect the responsiveness of leukocytes. The viscosity and cell-cell interaction with other leukocytes, hormones and cytokines of the surrounding cells can have a dramatic effect on shear stresses and the expression of cell surface receptors. Disruption to cell signalling pathways are minimised, and the responsiveness and integrity of cells is maintained.

ii) We avoid centrifugation a process known to affect cell reactivity, and avoid plating out cells on glass slides (used in the NBT test referred to in e.g. Tuskamoto et al.; see later).

iii) We stimulate the cells *in vitro* with PMA (but other equivalent inducers may be employed which are known to activate neutrophils in a manner consistent with the effect of natural stress exposure).

iv) We monitor the superoxide producing capacity of the cells in real time.

v) We monitor the rate of reactive oxygen production.

vi) As leukocytes release reactive oxygen species in response to stress, the stimulation allows us to see (like a differential equation) the capacity that the cells have to produce further reactive oxygen species.

vii) This takes into account the exposure to other stress mediators and

viii) makes the test sensitive to true stress;

ix) the reactivity of the cells is not altered by manipulation

5. The Examiner has asked for clarification of the measurements taken. One must have control measurements from control samples treated in identical manner to test samples taken from individuals exposed to a putative stressor. Then, coping capacity of such individuals for a putative stressor is assessed as below.

Control Sample(s) (where an individual or group of individuals are not subjected to the stressor of interest)

W= basal production of superoxide species in whole blood (without stimulant); basal production of superoxide may occur naturally as a result of circulating stimulants (hormones, cytokines, other cell to cell interaction) without the addition of an external stimulant such as PMA.

X = production of superoxide species with stimulant (e.g. with PMA).

Y = X-W = net additional superoxide species production above basal for control.

Test sample (where an individual or group of individuals has been exposed to a putative stressor)

$\mathbf{W_1}$= basal production of superoxide species in whole blood (without stimulant).

$\mathbf{X_1}$ = production of superoxide species with stimulant (e.g. with PMA).

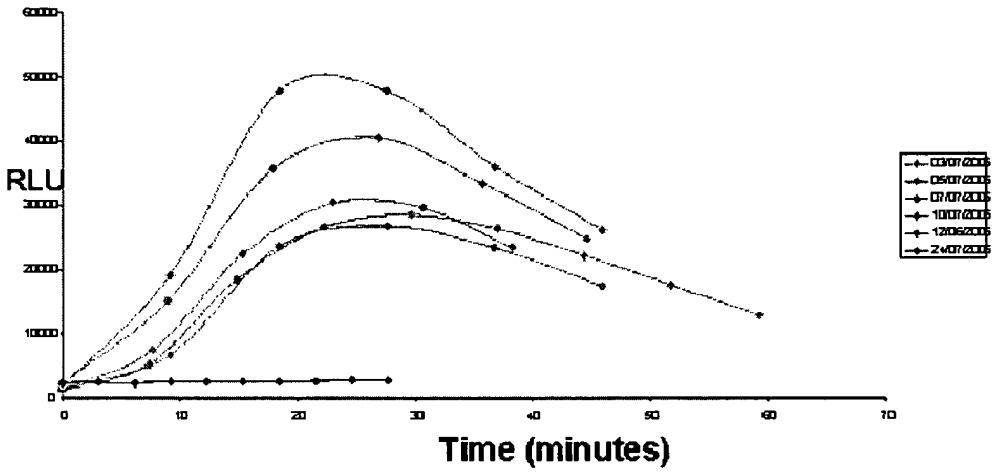
$\mathbf{Y_1 = X_1-W_1}$ = net additional superoxide species production above basal.

If $Y=Y_1$ Coping capacity excellent (i.e. stressor has no effect on the ability of blood cells (mainly neutrophils) to respond to the stimulant (e.g. PMA)

If $Y_1<Y$ capacity of cells for superoxide production has been reduced by stress exposure; residual capacity equated with coping capacity of individual.

In practice, superoxide production in the presence of stimulant will generally be observed over time as shown for the badger studies in Figure 1 of the patent application.

6. The graph below shows actual application of the same method to blood samples taken from a professional soccer player to assess coping capacity in the face of stressors of normal life of such a sportsman. All samples were taken at rest before training schedules.



RLU= Relative light units = Coping capacity of cells = coping capacity of the soccer player in training

The higher the response (coping capacity response measured in relative light units RLU), the better the ability to cope. The bottom line indicates a compromised immune system. In keeping with this, 3 days later the player developed an opportunistic infection. This compromised the player's ability to perform. This illustrates well how the method of the invention can be used in a real life situation with a sportsperson to objectively monitor coping capacity with time in a physiologically relevant manner, something that was just not achievable previously. Professional coaches have used such results, for example, to adjust training programmes and monitor effects of lifestyle and other external stressors.

7. The graphs below illustrate further actual application of the same method to blood samples taken from volunteers to assess coping capacity in the face of performing simple manoeuvres in two different types of cars (car type A and car type B; names withheld for reasons of confidentiality). This work was carried out by G. Shelton-Rayner (relevant information now provided is taken from Shelton-Rayner MPhil Transfer Report, Coventry University; Director of Studies, R. Mian; supervisor, D. W. Macdonald). Finger prick blood samples were taken before, just after and 45 minutes post tests. A mean set of results ± SEM were then calculated from Car series A data (n=21 subjects) and Car Series B data (n=18 subjects) and

displayed graphically as two line graphs as shown in Figures 2 and 3 below respectively.

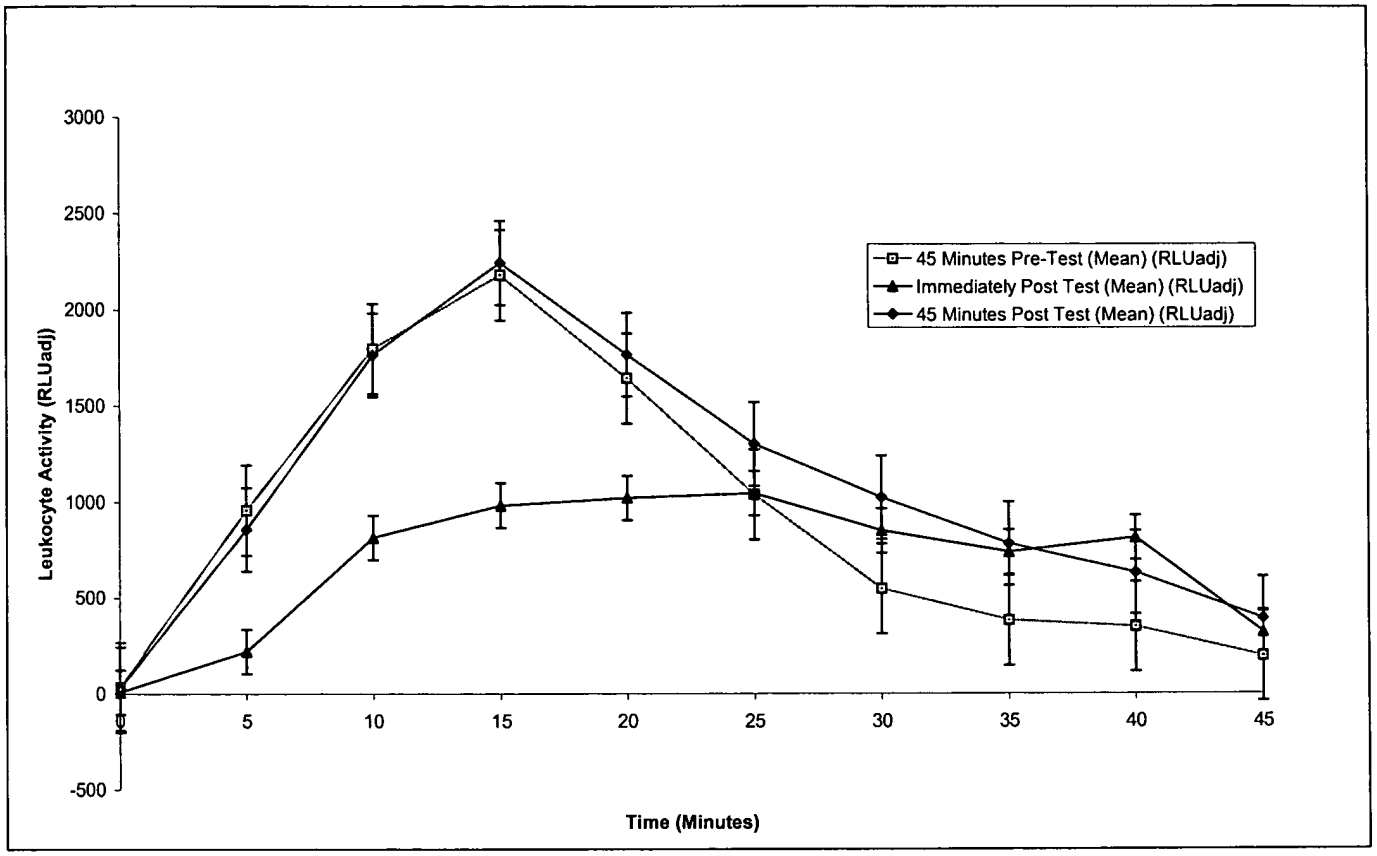


Figure 2. Line chart illustrating Mean Leukocyte Coping Capacity (RLU_{adj}) ± SEM 45minutes pre-test, immediately post test and 45 minutes post test after completing the driver environment protocol in the Car Series A (n=21).

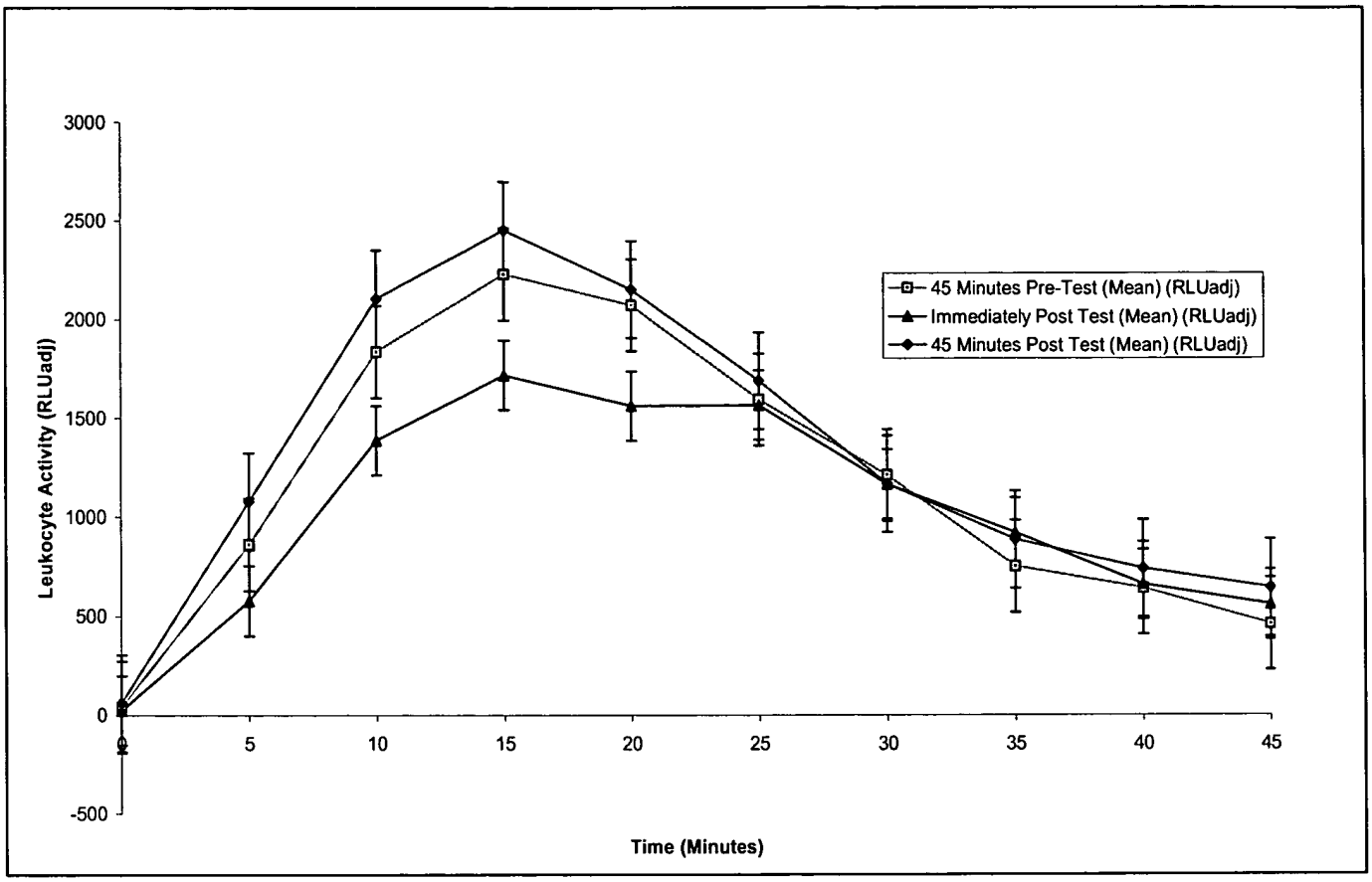


Figure 3. Line chart illustrating Mean Leukocyte Coping Capacity (RLU_{adj}) ± SEM 45minutes pre-test, immediately post test and 45 minutes post test after completing the driver environment protocol in the Car Series B(n=18).Names of manufactures withheld

Again, the higher the response (coping capacity response measured in relative light units, RLU), the better the ability to cope. The blue bottom line indicates a compromised immune system immediately after performing manoeuvres in both cars. Car A produced a significantly greater reduction in Leucocyte Coping Capacity (LCC) than car B, i.e. subjects were more able to cope with performing manoeuvres in car B. LCC responses returned to pre-test values 45 minutes after in both cases. This further illustrates well how the method of the invention can be used in a real life situation to objectively monitor coping capacity with time in a physiologically relevant manner,

8. These are just two ways in which our method is finding successful application. There are many others from environmental design to handling of wild animals. The latter is a particular interest of my co-inventor, David MacDonald, and was the original impetuswhich led to the method; he wished for a convenient, physiologically relevant method which could be used to assess exposure of wild animals to stress, which might be used even outside of the laboratory. At the time, I was working in the stress field and with others in my laboratory had made the finding that mental stress affects the number of stained activated neutrophils which can be observed in blood samples by the known NBT reduction test (Ellard, Castle & Mian (2001) Int. J. Physiol. 41, 93-100: The effect of a short term mental stressor on neutrophil activation). However, it was moving on to thinking about the performance of neutrophils *in vivo* under stress conditions which laid the foundations for the invention.

9. **Why is the blood suspension of cells so significant?** It is the answer to this question which is at the heart of the usefulness of our method and which has led to its success.

Leukocytes are 3 dimensional entities. Their ability to produce reactive oxygen species is altered by cell signalling pathways of other entities and cells.

Our test monitors the cellular capacity of leukocytes to produce superoxide radicals in

- Real time.
- It deliberately leaves the cells in contact with the circulating mediators of stress within blood. The leukocytes actively interact with other cell components and mediators released. Leukocytes (primarily neutrophils) have over 150 different receptors which can respond to a diverse range of factors, all of which are sensitive to stress.
- There is a 3 dimensional exposure to blood. Our test uses a drop of blood. The drop is maintained not spread on glass or preserved, the cells are allowed to interact with hormones (which can alter reactivity of the cells) other cells such as macrophages, other neutrophils, the haematocrit, red blood cells (whose viscosity alter during stress).

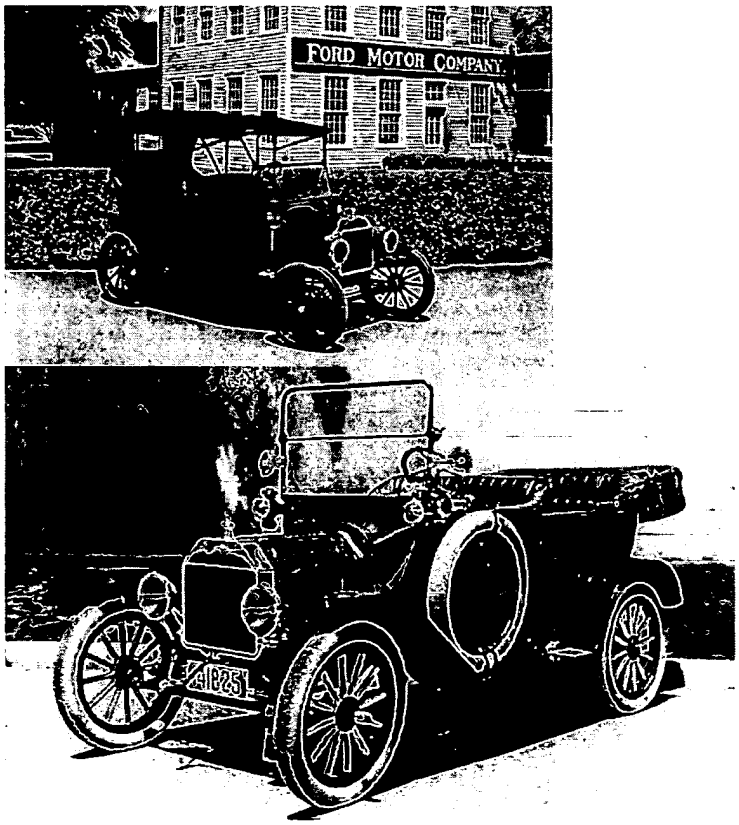
10. **How does our test differ from the NBT test used by Tuskamoto et al.?** The nitroblue tetrazolium (NBT) neutrophil reduction test has been known for many years. For such a test, blood is smeared onto a microscope slide and examined under the light microscope to determine the percentage of activated neutrophils. The NBT reacts with the granules present in activated neutrophils, forming intracytoplasmic deposits of formazan, which appear as dark blue granules under the light microscope. Such a test for scoring activated neutrophils is also described in Ellard et al. *ibid.* The NBT test in effect monitors morphological changes. In contrast, the method of the invention can monitor subtle changes in the functional capacity of leukocytes (primarily but not exclusively neutrophils) in real time to produce reactive oxygen species. Differences in leucocyte reactivity occur well before morphological differences become apparent. Moreover, Tsukamoto et al. did not employ PMA. They used *Staphylococcus aureas* (live bacteria) which will produce an entirely immune response and endotoxin which stimulates specific pathways distinct from PMA. PMA activates specific intracellular pathways, which are thought to mimic stress more closely than either live bacterial cells or endotoxin. In keeping with the very different nature of the method of Tsukamoto et al., their results for the Crowd II conditions were non-significant. This is expressly noted in the legend to their Figure 4.

11. The Examiner has indicated wish to move on to reliance principally on the Mikawa et al. paper. I can see the pattern, key words are correct (neutrophils, stimulation, superoxide production) but the Examiner is not reading these in the right context. I have welcomed being more educated about surgical operations in neonates, but the studies of Mikawa et al. do not form part of the technical background of the invention. I had no consideration of these at the time of devising of our test and even if I had done would have seen them as only relevant to those seeking to understand factors affecting recovery from surgery.

12. **Why is Mikawa et al. paper irrelevant to out test?** There is simply nothing in the disclosure of the Mikawa et al. paper which is remotely of interest in relation to out test; Mikawa et al. were not even assessing psychological stress. Perioperative stress (the subject of the Mikawa et al. paper) cannot be equated with psychological stress. The perioperative period is the time period surrounding a patient's surgical procedure; this commonly includes ward admission, anaesthesia, surgery and recovery. Perioperative, generally refers to the three phases of surgery: preoperative, intraoperative, postoperative. Subjects underwent **abdominal surgery** with **general**

anaesthesia (nitrous oxide and halothane in oxygen). Ventilation of patients was controlled. All of these interventions are well known to affect the activation of neutrophils. Mikawa et al. were studying an entirely different type of stressor (abdominal surgery stress). Mikawa et al. studied changes in the production of superoxide in isolated neutrophils, before, during and after **surgery.** I also highlight that Mikawa et al. used a fixed quantity of isolated neutrophils for stimulation. This meant centrifugation and thereby an alteration of sensitivity and exposure to conditions which would alter their responsiveness. I return to the key factor in the success of our test; we deliberately chose to keep neutrophils in their natural environment. This led us to use chemiluminescence measurement of stimulated superoxide production, a feature which is shared with the Mikawa et al. studies, but such measurement was long known and is not our invention.

13. Before our invention, study of the role of neutrophils in response to psychological stress had failed to consider the complexity of the stress process; this led to flawed tests. A car analogy may be helpful. If the cells are like cars, then two cells appear below.

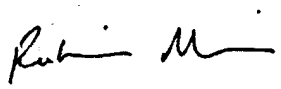


Both cars (cells) may look the same (histologically similar as might be revealed by NBT test). Imagine only one has an engine that works. Our test monitors the function of the car (cell) i.e. how well it performs. Performance of cars differs (0-60 in x seconds); we monitor the performance (functional ability) of the cells. Furthermore, the performance of one car (leukocyte) may impact on others. During stress, the ability of other cells and mediators will impact on the ability of leukocytes to generate reactive oxygen species; see pictures below.

Our test takes account of this by not isolating neutrophils as favoured previously by others. In summary, the performance of the cells is monitored in real time, using specific conditions, which gives a sensitive, user friendly, accurate measure of stress.

14. Interest in our test since it was first published in McLaren et al. J. Exp. Physiol. (2003) has been immense precisely because it represents a very different approach to looking at stress; it demanded a very different mind set from previous studies aimed at stress assessment and for the first time enabled a physiologically relevant objective measure of coping capacity for known psychological stressors. It has been the subject of further publications in peer-reviewed journal papers and, as indicated above, I was invited to review the technique in a book chapter in 'Stress and Health: New Research' (ed. K. Ovington, Nova Science Publications, N.Y). In further support that that our test is well outside the bounds of what others were actually doing and thinking in the field of psychological stress analysis at the time of our first patent application in November 2002, I can also attest that, as far as I am aware, it is the first test for objectively assessing psychological stress which has found real commercial interest.

15. All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such wilful false statements may jeopardise the validity of this declaration, the patent application, or any patents issuing thereon.



Date 1st November 2007

Rubina Mian